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(57) Abstract

We have identified a novel protein, named ALARM or δ–catenin, on the basis of its ability to bind to presenilin 1. ALARM contains 4 copies of the *arm* repeat and is expressed almost exclusively in brain tissue.

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ALARM RELATED PEPTIDES AND NUCLEIC ACIDS AND DIAGNOSIS USING THEM

Cross Reference To Related Application

This application claims priority from U.S. Provisional Application Serial No. 60/031,556 filed December 2, 1996, which is incorporated herein in its entirety.

Statement as to Federally Sponsored Research

This invention was made with Government support under AG06601 awarded by the National Institutes of Health. The Government has certain rights in the invention.

Background of the Invention

The invention is in the general field of proteins involved in Alzheimer's disease.

Various genes and gene products involved in the development of Alzheimer's disease have been identified. Neuritic plaques characteristic of the disease are composed of β-amyloid (Aβ), which are oligopeptides of about 40-43 amino acids in length derived from the β-amyloid precursor protein (βAPP). Mutations in the gene encoding βAPP are associated with some cases of familial Alzheimer's disease. Other cases of familial Alzheimer's disease have been associated with mutations in two other loci, presenilin-1 and presinilin-2.

Summary of the Invention

The invention is based on the discovery of a heretofore undescribed protein, which has been named ALARM or δ-catenin, on the basis of its interaction with presenilin 1. ALARM shows a striking sequence similarity to members of the *armadillo* (arm)-plakoglobin-β catenin

protein family. In addition, ALARM transcripts are confined almost exclusively to brain tissue.

In addition to the specific human ALARM sequences provided (or cross-referenced) herein, molecules relevant to the invention include fragments of those sequences and related polypeptides, non-peptide mimetics, and nucleic acid sequences. The invention also includes antibodies to ALARM polypeptides. These polypeptides, as well as nucleic acid encoding them, can be used for a variety of diagnostic and therapeutic applications.

In one aspect the invention features a substantially pure vertebrate ALARM polypeptide, e.g, an ALARM polypeptide from a mammal such as the human ALARM polypeptide shown in Fig. 1.

By "protein" and "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation).

Polypeptides include, but are not limited to: recombinant polypeptides, natural polypeptides, and synthetic polypeptides as well as polypeptides which are preproteins or proproteins.

One way to ascertain purity of a preparation is by per cent dry weight. Generally, useful preparations are at least 60% by weight (dry weight) the compound of interest, i.e., an ALARM polypeptide. Preferably the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight the compound of interest. Purity can be measured by any appropriate standard method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis. A "mature human ALARM" is the amino acid sequence shown in Fig. 1.

Polypeptides substantially identical to mature human ALARM may have an amino acid sequence which is at

least 85%, preferably 90%, and most preferably 95% or even 99% identical to the amino acid sequence of the ALARM polypeptide of the Fig. 1. When assessing sequence identity of polypeptides, the length of the reference polypeptide sequence will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids. For nucleic acids, the length of the reference nucleic acid sequence will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides.

Sequence identity can be measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705).

In the case of polypeptide sequences which are less than 100% identical to a reference sequence, the non-identical positions are preferably, but not necessarily, conservative substitutions for the reference sequence. Conservative substitutions typically include substitutions within the following groups: glycine and alanine; valine, isoleucine, and leucine; aspartic acid and glutamic acid; asparagine and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine.

Where a particular polypeptide is said to have a specific percent identity to a reference polypeptide of a defined length, the percent identity is relative to the reference peptide. Thus, a peptide that is 50% identical to a reference polypeptide that is 100 amino acids long can be a 50 amino acid polypeptide that is completely identical to a 50 amino acid long portion of the reference polypeptide. It might also be a 100 amino acid

long polypeptide which is 50% identical to the reference polypeptide over its entire length. Of course, many other polypeptides will meet the same criteria.

Polypeptides corresponding to one or more domains of ALARM are also within the scope of the invention. Thus, also featured is a polypeptide including at least one antigenic determinant of ALARM, a polypeptide comprising at least one copy of the 42 amino acid *arm* repeat in the ALARM polypeptide, or a polypeptide comprising a βAPP binding domain of ALARM. Preferred polypeptides are those which are soluble under normal physiological conditions.

The polypeptides of the invention can be expressed fused to another polypeptide, e.g., a marker polypeptide or fusion partner. For example, the polypeptide can be fused to a hexa-histidine tag to facilitate purification of bacterially expressed protein or a hemagglutinin tag to facilitate purification of protein expressed in eukaryotic cells.

In another aspect, the invention also features a substantially pure polypeptide which includes a first portion and a second portion; the first portion includes an ALARM polypeptide and the said second portion includes a detectable marker. The first portion can be either a full-length form of ALARM or one or more domains thereof. The first portion is fused to an unrelated protein or polypeptide (i.e., a fusion partner) to create a fusion protein.

The invention also includes a pharmaceutical composition which includes an ALARM polypeptide.

In still another aspect the invention features a recombinant nucleic acid encoding an ALARM polypeptide. In one preferred embodiments the nucleic acid encodes a soluble ALARM polypeptide.

The invention also features isolated nucleic acids encoding polypeptides corresponding to one or more domains of ALARM or ALARM-related polypeptides discussed above. ALARM-encoding nucleotides can include the nucleic acids shown in Fig. 1, e.g., nucleotides 366-2636 of Fig. 1. Also encompassed within the invention are nucleic acid sequences that encode forms of ALARM in which sequences are altered or deleted.

By "isolated nucleic acid" is meant nucleic acid that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. Thus, a recombinant nucleic acid could include some or all of the 5' non-coding (e.,g., promoter) sequences which are immediately contiguous to the coding sequence. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus, such as a retrovirus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

Nucleic acid sequences substantially identical to human ALARM sequences have a nucleotide sequence which is at least 85%, preferably 90%, and most preferably 95% or even 99% identical to the amino acid sequence of the ALARM polypeptide of the Fig. 1. For nucleic acids, the length of the reference nucleic acid sequence will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides.

Also within the invention are nucleic acids encoding hybrid proteins in which a portion of ALARM or a portion (e.g., one or more domains) thereof is fused to an unrelated protein or polypeptide (i.e., a fusion partner) to create a fusion protein.

The nucleic acid can be isolated either as a matter of purity or by including in it in DNA that is a non-naturally occurring molecule; for example, the DNA is not immediately contiguous with both of the sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. Thus, a recombinant nucleic acid could include some or all of the 5' non-coding (e.,g., promoter) sequences which are immediately contiguous to the coding sequence. Other examples are a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

The nucleic acids of the invention include nucleic acids encoding ALARM polypeptides fused to a polypeptide which facilitates secretion, e.g., a secretory sequence. Such a fused protein is typically referred to as a preprotein. The secretory sequence can be removed by the host cell to form the mature protein. Also within the invention are nucleic acids that encode mature ALARM fused to a polypeptide sequence to produce an inactive preprotein. Preproteins can be converted into the active form of the protein by removal of the inactivating sequence.

The invention also encompasses nucleic acids that hybridize under stringent conditions to a nucleic acid encoding an ALARM polypeptide. "Stringent conditions" means hybridization at 50°C in Church buffer (7% SDS, 0.5% $NaHPO_4$, 1mM EDTA,1%BSA) and washing at 50°C in 2x SSC. The hybridizing portion of the hybridizing nucleic acids are preferably 20, 30, 50, or 70 bases long. Preferably, the hybridizing portion of the hybridizing nucleic acid is 95% or even 98% identical to the sequence of a portion of a nucleic acid encoding an ALARM polypeptide. Hybridizing nucleic acids of the type described above can be used as a cloning probe, a primer (e.g., a PCR primer), or a diagnostic probe. Preferred hybridizing nucleic acids encode a polypeptide having some or all of the biological activities possessed by naturally-occurring ALARM. Hybridizing nucleic acids can be splice variants encoded by one of the ALARM genes described herein. Thus, they may encode a protein which is shorter or longer than the various forms of ALARM described herein. Hybridizing nucleic acids may also encode proteins which are related to ALARM (e.g, proteins encoded by genes which include a portion having a relatively high degree of identity to an ALARM gene described herein).

The term "nucleic acid" encompasses both RNA and DNA, including cDNA, genomic DNA, and synthetic (e.g., chemically synthesized) DNA. The nucleic acid may be double-stranded or single-stranded. Where single-stranded, the nucleic acid may be the sense strand or the antisense strand.

In yet another aspect, the invention features vectors which include a nucleic acid of the invention. In one preferred embodiment, the nucleic acid of the invention is properly positioned for expression.

By "positioned for expression" is meant that the selected DNA molecule is positioned adjacent to one or more sequence elements which direct transcription and/or translation of the sequence such that the sequence elements can control transcription and/or translation of the selected DNA (i.e., the selected DNA is operably associated with the sequence elements). Such operably associated elements can be used to facilitate the production of an ALARM polypeptide.

In a still further aspect, the invention features transformed cells harboring a nucleic acid encoding ALARM sequences discussed above.

By "transformed cell" is meant a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding (as used herein) ALARM polypeptide.

The invention also features purified antibodies which specifically bind an ALARM protein or polypeptide.

By "purified antibody" is meant an antibody which is at least 60%, by dry weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by dry weight, antibody.

By "specifically binds" is meant an antibody that recognizes and binds to and forms a complex with, a particular antigen, e.g., ALARM polypeptide, but which does not substantially recognize and bind to other molecules in a sample, e.g., a biological sample, which naturally includes ALARM.

The invention also features a method of diagnosing in a mammal, e.g., a human subject, an increased likelihood of, inclination toward, or susceptibility to developing a disease, in which a mutant form of the ALARM protein is a causative agent. The same method is also

used to diagnose the ability of a mammal, e.g., a human, to transmit to future generations a mutant form of a protein which is a causative agent of a disease. The method involves analyzing the DNA of the mammal to determine the presence or absence of a mutation in a gene for an ALARM protein, the presence of such a mutation indicating the increased likelihood. Preferably the DNA is analyzed by amplifying the DNA with, e.g., the polymerase chain reaction, and identifying mutations in the DNA by use of the single-strand conformation polymorphism (SSCP) technique, as used and described herein, or by direct DNA sequencing.

In another aspect, the invention includes a method of inhibiting expression of an ALARM gene comprising administering to a cell containing an ALARM transcript an anti-sense ALARM oligonucleotide.

The invention also includes a method of detecting presenilin 1 in a sample, e.g., a sample taken from a human, comprising contacting the sample with an ALARM polypeptide. The sample can be from, e.g., cerebrospinal fluid.

In another aspect, the invention includes a method of diagnosing in a human subject a disease in which a mutant form of a protein which interacts with ALARM is a causative agent. The method includes analyzing a sample of fluid from the human subject to determine the presence or absence of the ALARM-interacting protein.

The invention further includes a method of diagnosing in a human subject an increased likelihood of developing or transmitting to future generations a disease in which a mutant form of a human ALARM is a causative agent. The method includes analyzing the DNA of the subject to determine the presence or absence of a mutation in a gene for an ALARM protein, the presence of

such a mutation indicating the increased likelihood of transmitting the disease. The method can include, e.g., amplifying the DNA of the subject, DNA sequencing, or identifying a single strand conformation polymerism.

The invention also includes a probe or primer comprising a substantially purified single-stranded oligonucleotide, e.g., a DNA oligonucleotide, wherein the oligonucleotide contains a region which is identical to the sequence of a six-nucleotide, single-stranded segment of a gene encoding a mutant form of a human ALARM, wherein the segment comprising part or all of the mutation.

In yet another aspect, the invention includes a method of detecting an ALARM-containing complex in a biological sample by contacting the sample with an ALARM protein or an ALARM antibody and determining whether the ALARM protein or antibody binds to a component of the sample.

In a further aspect, the invention includes a method of diagnosing altered levels, e.g., lower or altered levels, of presenilin 1 in a sample by contacting the sample with ALARM and determining whether the sample contains presenilin 1 that binds to ALARM.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions, will control. In addition, the materials,

methods, and examples are illustrative only and are not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed descriptions, and from the claims.

Brief Description of the Figures

Figs. 1A-1E are a schematic representation of the predicted nucleotide and amino acid sequence of the human ALARM protein.

Fig. 2 is a schematic representation of the ALARM *arm* repeats and their homology to the *Drosophila arm* sequence.

Fig. 3 is a schematic representation of the ALARM and pp120 amino acid sequences.

Fig. 4 is a schematic representation of the ALARM and γ catenin amino acid sequences.

Detailed Description

Previously described genes encoding proteins involved in Alzheimer's disease include βAPP, which was isolated as the cellular protein giving rise to the polypeptide fragments found in the Aβ plaques characteristic of Alzheimer's disease (reviewed in Selkoe, Ann. Rev. Cell Biol. 10:373, 1994), as well as presenilin 1 and presenilin 2, which were identified as cellular genes altered in cases of familial Alzheimer's disease (Sherrington et al., Nature 375:754, 1995; Levy-Lahad et al., Science 259:970, 1995; Rogaev et al., Nature 376:207, 1995).

βAPP, presenilin-1, and presenilin-2 all encode transmembrane proteins. The protein encoded by βAPP has a type I single transmembrane segment (Selkoe, supra), while the presenilin 1 and presenilin 2 polypeptides have seven putative transmembrane segments (Sherrington et

al., supra, 1995; Levy-Lahad et al., Science 269:973, 1995; Rogaev et al., supra). In addition, presenilin 1 and 2 are homologous to the *sel-12* gene in the nematode, *C. elegans*, which likewise encodes a protein with seven putative transmembrane segments (Leviatan et al., Nature 377:351, 1995; Grant et al., Genetics 143:237, 1996). The *sel-12* gene was identified as a suppressor of defects in the *lin-12* locus, which encodes a type I transmembrane protein (Sundaram et al., Genetics 135:765, 1993; Yochem et al. Nature 335:547, 1988). Based in part on this similarity, a model has been proposed in which the βAPP protein binds to the presenilin-1 or presenilin 2 gene product (Dewji et al., Science 271:159, 1996).

Until the present discovery, however, little was known about how products of the presenilin 1 and presenilin 2 genes interacted with each other, with other proteins, or whether they participated in any known signal transduction pathways. We have used the two-hybrid yeast system to identify a novel human protein on the basis of its interaction with the single hydrophilic loop region of presenilin 1. The interacting protein contains multiple copies of an amino acid repeat sequence first described in the *armadillo* (*arm*) gene in the fruit fly, *Drosophila melanogaster* (Riggleman et al., Genes Develop. 3:96, 1989). Proteins with the *arm* repeat have been subsequently identified in several other proteins, including plakoglobin, β-catenin, and p120 (Peifer et al., J. Cell Biol. 118:681, 1992); Reynolds et al., Oncogene 7:2439, 1992). As other members of this family have been localized to the adherens junction, the new protein has been named ALARM, for adherens-junction linked *arm* protein. Alternatively, it can also be called δ-catenin, since it shows homology to known members of the catenin protein family.

Two functions have previously been ascribed to members of the *arm* family. First, evidence from diverse organisms suggests that *arm* is involved in the *Wnt* signal transduction pathway. *Wnt* homologs in a variety of organisms have been associated with signalling functions during animal development. In general, *Wnt* functions act so that groups of cells maintain the same identity as neighboring cells. Thus, in *Drosophila* the *Wnt* homolog, *wingless* (*wg*), acts to maintain *engrailed* expression in adjacent group of cells. (DiNardo et al., Nature 332:604, 1988; Martinez-Aria et al., Development 103:157, 1988). Similarly, addition of *wg*, to *Drosophila* embryos increases the level of *arm* protein (Riggleman et al., Cell 63:549, 1990). This interaction is mediated through the binding of *wg* to cell-surface receptors encoded by members of the *frizzled* (*Dfz*) gene family (Bhanot et al., Nature 382:225, 1996). Other *Drosophila* genes involved in the *Wng* signalling pathway include *dishevelled* (*dsh*) and *zeste-white 3* (*zw3*) (see Bhanot et al., supra).

In *Xenopus laevis* embryos, ectopic expression of β-catenin results in a phenotype similar to that caused by mutations in some member of the *Wnt* family (Guger et al., Dev. Biol 172:115-25). In mammalian cells, *Wnt-1* expression results in the accumulation of β-catenin and plakoglobin (Hinck et al., J. Cell Biol. 124:729, 1994).

β catenin also forms a complex with the transcription factor LEF-1, and this complex localizes to the nucleus. (Behrens et al., Nature 382:638, 1996). Thus, a combination of genetic and biochemical studies suggest *arm* family members may be involved in transducing signals from the cell-surface to the nucleus in the *Wnt* pathway.

The second function in which members of the *arm* family have been implicated is promotion of cell adhesion. Plakoglobin, β catenin, and p120 all associate

with the cytoplasmic domains of the calcium-dependent cell-cell adhesion proteins called cadherins (Daniel et al., Mol Cell. Biol. 15:4819, 1995); Shibamoto et al., J. Cell. Biol. 128:949, 1995). p120 is thought to associate with E-cadherin via E-cadherin's carboxyl terminus (Shibamoto et al., supra). Similarly, *arm* proteins have been localized to the cytoplasmic surface of cells and colocalize with actin. (Riggleman et al., Cell 63:549, 1990).

The present invention for the first time suggests members of the *arm* family are involved in the pathology of Alzheimer's disease.

ALARM Polypeptides, Proteins and Nucleic Acid Sequences

The invention encompasses, but is not limited to, ALARM proteins and polypeptides that are functionally related to ALARM encoded by the nucleotide sequence of Fig. 1. Functionally related proteins and polypeptides include any protein or polypeptide sharing a functional characteristic with ALARM, e.g., the ability to bind to presenilin 1. Such functionally related ALARM polypeptides include, but are not limited to, additions or substitutions of amino acid residues within the amino acid sequence encoded by the ALARM sequences described herein which result in a silent change, thus producing a functionally equivalent gene product. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved.

For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine,

tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

ALARM polypeptides and proteins of the invention can be made by altering nucleic acid sequences encoding ALARM polypeptides. While random mutations can be made to ALARM DNA (using random mutagenesis techniques well known to those skilled in the art) and the resulting mutant ALARM proteins can be tested for activity, site-directed mutations of the ALARM coding sequence can be engineered (using site-directed mutagenesis techniques well known to those skilled in the art) to generate mutant ALARMs.

To design variant ALARM polypeptides which may be altered in their function, *e.g.*, in their ability to bind to presenilin 1, it is useful to distinguish between conserved positions and variable positions. Conserved positions are those in which the amino acid in an ALARM protein from another organism as in the same position as it is in the human ALARM protein.

To preserve ALARM function, it is preferable that conserved residues are not altered. Moreover, alteration of non-conserved residues are preferably conservative alterations, e.g., a basic amino acid is replaced by a different basic amino acid. To produce altered function variants, it is preferable to make non-conservative changes at variable and/or conserved positions. Deletions at conserved and variable positions can also be used to create altered function variants.

Other mutations to the ALARM coding sequence can be made to generate ALARMs that are better suited for expression, *e.g.*, scaled up expression, in a selected host cell. For example, potential N-linked glycosylation sites can be altered or eliminated to achieve, for

example, expression of a homogeneous product that is more easily recovered and purified from yeast hosts which are known to hyperglycosylate N-linked sites. To this end, a variety of amino acid substitutions at one or both of the first or third amino acid positions of any one or more of the glycosylation recognition sequences which occur (in N-X-S or N-X-T), and/or an amino acid deletion at the second position of any one or more of such recognition sequences, will prevent glycosylation at the modified tripeptide sequence. (See , e.g., Miyajima et al., Embo J. 5:1193, 1986).

Preferred ALARM polypeptides are those polypeptides, or variants thereof, which bind to presenilin 1 polypeptides. In determining whether a particular ALARM polypeptide or variant thereof binds to presenilin 1, one can use any assay techniques disclosed herein or in referenced publications. Preferred ALARM polypeptides and variants have 20%, 40%, 50%, 75%, 80%, or even 90% of the activity of the full-length, mature human form of ALARM described herein. Such comparisons are generally based on equal concentrations of the molecules being compared. The comparison can also be based on the amount of protein or polypeptide required to reach 50% of the maximal stimulation obtainable.

Also within the invention are fusion proteins in which a portion (e.g., one or more domains) of ALARM is fused to an unrelated protein or polypeptide (i.e., a fusion partner) to create a fusion protein. The fusion partner can be a moiety selected to facilitate purification, detection, or solubilization, or to provide some other function. Fusion proteins are generally produced by expressing a hybrid gene in which a nucleotide sequence encoding all or a portion of ALARM is joined in-frame to a nucleotide sequence encoding the fusion partner.

In general, ALARM proteins according to the invention can be produced by transformation (transfection, transduction, or infection) of a host cell with all or part of an ALARM-encoding DNA fragment (e.g., the ALARM DNA described herein) in a suitable expression vehicle. Suitable expression vehicles include: plasmids, viral particles, and phage. For insect cells, baculovirus expression vectors are suitable. The entire expression vehicle, or a part thereof, can be integrated into the host cell genome. In some circumstances, it is desirable to employ an inducible expression vector, e.g., the LACSWITCH™ Inducible Expression System (Stratagene; LaJolla, CA).

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems can be used to provide the recombinant protein. The precise host cell used is not critical to the invention. The ALARM protein can be produced in a prokaryotic host (e.g., *E. coli* or *B. subtilis*) or in a eukaryotic host (e.g., *Saccharomyces* or *Pichia*; mammalian cells, e.g., COS, NIH 3T3 CHO, BHK, 293, or HeLa cells; or insect cells).

Proteins and polypeptides can also be produced by plant cells. For plant cells viral expression vectors (e.g., cauliflower mosaic virus and tobacco mosaic virus) and plasmid expression vectors (e.g., Ti plasmid) are suitable. Such cells are available from a wide range of sources (e.g., the American Type Culture Collection, Rockland, MD; also, see, e.g., Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1994). The methods of transformation or transfection and the choice of expression vehicle will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al. (*Current Protocols in Molecular Biology*, John Wiley &

Sons, New York, 1994); expression vehicles may be chosen from those provided, e.g., in *Cloning Vectors*: A Laboratory Manual (P.H. Pouwels et al., 1985, Supp. 1987).

The host cells harboring the expression vehicle can be cultured in conventional nutrient media adapted as need for activation of a chosen gene, repression of a chosen gene, selection of transformants, or amplification of a chosen gene.

One preferred expression system is the mouse 3T3 fibroblast host cell transfected with a pMAMneo expression vector (Clontech, Palo Alto, CA). pMAMneo provides an RSV-LTR enhancer linked to a dexamethasone-inducible MMTV-LTR promotor, an SV40 origin of replication which allows replication in mammalian systems, a selectable neomycin gene, and SV40 splicing and polyadenylation sites. DNA encoding an ALARM protein would be inserted into the pMAMneo vector in an orientation designed to allow expression. The recombinant ALARM protein would be isolated as described below. Other preferable host cells that can be used in conjunction with the pMAMneo expression vehicle include COS cells and CHO cells (ATCC Accession Nos. CRL 1650 and CCL 61, respectively).

ALARM polypeptides can be produced as fusion proteins. For example, the expression vector pUR278 (Ruther et al., EMBO J. 2:1791, 1983), can be used to create lacZ fusion proteins. The pGEX vectors can be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can be easily purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the

cloned target gene product can be released from the GST moiety.

In an insect cell expression system, *Autographa californica* nuclear polyhidrosis virus (AcNPV), which grows in *Spodoptera frugiperda* cells, is used as a vector to express foreign genes. An ALARM coding sequence can be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter, e.g., the polyhedrin promoter. Successful insertion of a gene encoding an ALARM polypeptide or protein will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat encoded by the polyhedrin gene). These recombinant viruses are then used to infect spodoptera frugiperda cells in which the inserted gene is expressed (see, e.g., Smith et al., J. Virol. 46:584, 1983; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems can be utilized. In cases where an adenovirus is used as an expression vector, the ALARM nucleic acid sequence can be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene can then be inserted into the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion into a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing an ALARM gene product in infected hosts (see, e.g., Logan, Proc. Natl. Acad. Sci. USA 81:3655, 1984).

Specific initiation signals may also be required for efficient translation of inserted nucleic acid sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire

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native ALARM gene or ALARM cDNA, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. In other cases, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators (Bittner et al., Methods in Enzymol. 153:516, 1987).

In addition, a host cell may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in a specific, desired fashion. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product can be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and in particular, choroid plexus cell lines.

Alternatively, an ALARM protein can be produced by a stably-transfected mammalian cell line. A number of

vectors suitable for stable transfection of mammalian cells are available to the public, see, e.g., Pouwels et al. (supra); methods for constructing such cell lines are also publicly available, e.g., in Ausubel et al. (supra). In one example, cDNA encoding the ALARM protein is cloned into an expression vector that includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, the ALARM protein-encoding gene into the host cell chromosome is selected for by including 0.01-300 μM methotrexate in the cell culture medium (as described in Ausubel et al., supra). This dominant selection can be accomplished in most cell types.

Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene. Methods for selecting cell lines bearing gene amplifications are described in Ausubel et al. (supra); such methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. DHFR-containing expression vectors commonly used for this purpose include pCVSEII-DHFR and pAdD26SV(A) (described in Ausubel et al., supra). Any of the host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR cells, ATCC Accession No. CRL 9096) are among the host cells preferred for DHFR selection of a stably-transfected cell line or DHFR-mediated gene amplification.

A number of other selection systems can be used, including but not limited to the herpes simplex virus thymidine kinase, hypoxanthine-guanine phosphoribosyl-transferase, and adenine phosphoribosyltransferase genes can be employed in tk, hgprt, or aprt cells, respectively. In addition, gpt, which confers resistance to mycophenolic acid (Mulligan et al., Proc. Natl. Acad. Sci. USA 78:2072, 1981); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., J.

Mol. Biol. 150:1, 1981); and hygro, which confers resistance to hygromycin (Santerre et al., Gene 30:147, 1981), can be used.

Alternatively, any fusion protein can be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described in Janknecht et al., Proc. Natl. Acad. Sci. USA, 88:8972, 1991), allows for the ready purification of non-denatured fusion proteins expressed in human cell lines. In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni^{2+} nitriloacetic acid-agarose columns, and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

Alternatively, ALARM or a portion thereof, can be fused to an immunoglobulin Fc domain. Such a fusion protein can be readily purified using an affinity column.

ALARM proteins and polypeptides can also be expressed in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, and non-human primates, e.g., baboons, monkeys, and chimpanzees, can be used to generate ALARM-expressing transgenic animals.

Any technique known in the art can be used to introduce an ALARM transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al., Proc. Natl. Acad. Sci., USA 82:6148, 1985); gene targeting into embryonic stem cells (Thompson et al.,

Cell 56:313, 1989); and electroporation of embryos (Lo, Mol. Cell. Biol. 3:1803, 1983).

The present invention provides for transgenic animals that carry the ALARM transgene in all their cells, as well as animals that carry the transgene in some, but not all of their cells, i.e., mosaic animals. The transgene can be integrated as a single transgene or in concatamers, e.g., head-to-head tandems or head-to-tail tandems The transgene can also be selectively introduced into and activated in a particular cell type (Lasko et al., Proc. Natl. Acad. Sci. USA 89:6232, 1992). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

When it is desired that the ALARM transgene be integrated into the chromosomal site of the endogenous ALARM gene, gene targeting is preferred. Briefly, when such a technique is to be used, vectors containing some nucleotide sequences homologous to an endogenous ALARM gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous gene. The transgene also can be selectively introduced into a particular cell type, thus inactivating the endogenous ALARM gene in only that cell type (Gu et al., Science 265:103, 1984). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

Once transgenic animals have been generated, the expression of the recombinant ALARM gene can be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques

to analyze animal tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, *in situ* hybridization analysis, and RT-PCR. Samples of ALARM gene-expressing tissue, also can be evaluated immunocytochemically using antibodies specific for the ALARM transgene product.

Once the recombinant ALARM protein is expressed, it is isolated. Secreted forms can be isolated from the culture media, while non-secreted forms must be isolated from the host cells. Proteins can be isolated by affinity chromatography. In one example, an anti-ALARM protein antibody (e.g., produced as described herein) is attached to a column and used to isolate the ALARM protein. Lysis and fractionation of ALARM protein-harboring cells prior to affinity chromatography can be performed by standard methods (see, e.g., Ausubel et al., supra). Alternatively, an ALARM fusion protein, for example, an ALARM-maltose binding protein, an ALARM-β-galactosidase, or an ALARM-trpE fusion protein, can be constructed and used for ALARM protein isolation (see, e.g., Ausubel et al., supra; New England Biolabs, Beverly, MA).

Once isolated, the recombinant protein can, if desired, be further purified, e.g., by high performance liquid chromatography using standard techniques (see, e.g., Fisher, *Laboratory Techniques In Biochemistry And Molecular Biology*, eds., Work and Burdon, Elsevier, 1980).

Polypeptides of the invention, particularly short ALARM fragments, can also be produced by chemical synthesis (e.g., by the methods described in *Solid Phase*

Peptide Synthesis, 2nd ed., 1984 The Pierce Chemical Co., Rockford, IL).

These general techniques of polypeptide expression and purification can also be used to produce and isolate useful ALARM fragments or analogs (described herein).

The invention also features proteins which interact with ALARM and are involved in the function of ALARM. Also included in the invention are the genes encoding these interacting proteins. Interacting proteins can be identified using methods known to those skilled in the art. One method suitable method is the "two-hybrid system," detects protein interactions *in vivo* (Chien et al., Proc. Natl. Acad. Sci. USA, 88:9578, 1991). A kit for practicing this method is available from Clontech (Palo Alto, CA).

Anti-ALARM Antibodies

Human ALARM proteins and polypeptides (or immunogenic fragments or analogs) can be used to raise antibodies useful in the invention; such polypeptides can be produced by recombinant or peptide synthetic techniques (see, e.g., *Solid Phase Peptide Synthesis*, supra; Ausubel et al., supra). In general, the peptides can be coupled to a carrier protein, such as KLH, as described in Ausubel et al., supra, mixed with an adjuvant, and injected into a host mammal. Antibodies can be purified by peptide antigen affinity chromatography.

In particular, various host animals can be immunized by injection with an ALARM protein or polypeptide. Host animals include rabbits, mice, guinea pigs, and rats. Various adjuvants can be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum

hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.

Antibodies within the invention include monoclonal antibodies, polyclonal antibodies, humanized or chimeric antibodies, single chain antibodies, Fab fragments, $F(ab')_2$ fragments, and molecules produced using a Fab expression library.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, can be prepared using the ALARM proteins described above and standard hybridoma technology (see, e.g., Kohler et al., Nature 256:495, 1975; Kohler et al., Eur. J. Immunol. 6:511, 1976; Kohler et al., Eur. J. Immunol. 6:292, 1976; Hammerling et al., In Monoclonal Antibodies and T Cell Hybridomas, Elsevier, NY, 1981; Ausubel et al., supra).

In particular, monoclonal antibodies can be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture such as described in Kohler et al., Nature 256:495, 1975, and U.S. Patent No. 4,376,110; the human B-cell hybridoma technique (Kosbor et al., Immunology Today 4:72, 1983; Cole et al., Proc. Natl. Acad. Sci. USA 80:2026, 1983), and the EBV-hybridoma technique (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96, 1983). Such antibodies can be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the Mab of this invention may be cultivated *in vitro* or *in vivo*. The ability to produce high titers of mAbs *in*

vivo makes this the presently preferred method of production.

Once produced, polyclonal or monoclonal antibodies are tested for specific ALARM recognition by Western blot or immunoprecipitation analysis by standard methods, e.g., as described in Ausubel et al., supra.

Preferably, antibodies of the invention are produced using fragments of the ALARM protein which lie outside highly conserved regions and appear likely to be antigenic, by criteria such as high frequency of charged residues. In one specific example, such fragments are generated by standard techniques of PCR, and are then cloned into the PGEX expression vector (Ausubel et al., supra). Fusion proteins are expressed in *E. coli* and purified using a glutathione agarose affinity matrix as described in Ausubel, et al., supra.

Antisera can be raised by injections in a series, preferably including at least three booster injections. In some cases it may be desirable to minimize the potential problems of low affinity or specificity of antisera. In such circumstances involving fusion proteins, two or three ALARM fusion proteins can be generated for each protein, and each fusion protein can be injected into at least two rabbits.

Antisera is also checked for its ability to immunoprecipitate recombinant ALARM proteins or control proteins, such as glucocorticoid receptor, CAT, or luciferase.

The antibodies can be used, for example, in the detection of the ALARM in a biological sample as part of a diagnostic assay. Antibodies also can be used in a screening assay to measure the effect of a candidate compound on expression or localization of ALARM. Additionally, such antibodies can be used in conjunction with the gene therapy techniques described to, for

example, evaluate the normal and/or engineered ALARM-expressing cells prior to their introduction into the patient. Such antibodies additionally can be used in a method for inhibiting abnormal ALARM activity.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., Proc. Natl. Acad. Sci., 81:6851, 1984; Neuberger et al., Nature, 312:604, 1984; Takeda et al., Nature, 314:452, 1984) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine Mab and a human immunoglobulin constant region.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; and U.S. Patents 4,946,778 and 4,704,692) can be adapted to produce single chain antibodies against an ALARM protein or polypeptide. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments that recognize and bind to specific epitopes can be generated by known techniques. For example, such fragments include but are not limited to $F(ab')_2$ fragments that can be produced by pepsin digestion of the antibody molecule, and Fab fragments that can be generated by reducing the disulfide bridges of $F(ab')_2$ fragments. Alternatively, Fab expression libraries can be constructed (Huse et al., Science 246:1275, 1989) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibodies to the ALARM can, in turn, be used to generate anti-idiotype antibodies that resemble a portion of ALARM using techniques well known to those skilled in the art (see, e.g., Greenspan et al., FASEB J. 7:437, 1993; Nissinoff, J. Immunol. 147:2429, 1991). For example, antibodies that bind to ALARM and competitively inhibit the binding of a ligand of ALARM can be used to generate anti-idiotypes that resemble a ligand binding domain of ALARM and, therefore, bind and neutralize a ligand of ALARM. Such neutralizing anti-idiotypic antibodies or Fab fragments of such anti-idiotypic antibodies can be used in therapeutic regimens.

ALARM Oligonucleotide Diagnostic and Therapeutic Agents

Oligonucleotide therapeutic agents can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other appended groups such as peptides (e.g., for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (as described, e.g., in Letsinger et al., Proc. Natl. Acad. Sci. USA 86:6553, 1989; Lemaitre et al., Proc. Natl. Acad. Sci. USA 84:648, 1987; PCT Publication No. WO 88/09810) or the blood-brain barrier (see, e.g., PCT Publication No. WO 89/10134), or hybridization-triggered cleavage agents (see, e.g., Krol et al., BioTechniques 6:958, 1988), or intercalating agents (see, e.g., Zon, Pharm. Res. 5:539, 1988). To this end, the oligonucleotide can be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, or hybridization-triggered cleavage agent.

The oligonucleotide may comprise at least one modified base moiety which is selected from the group including, but not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethyl-aminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-theouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 2-(3-amino-3-N-2-carboxypropl) uracil, (acp3)w, and 2,6-diaminopurine.

The oligonucleotide may also comprise at least one modified sugar moiety selected from the group including, but not limited to, arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal, or an analog of any of these backbones.

In yet another embodiment, the oligonucleotide is an α-anomeric oligonucleotide. An α-anomeric oligonucleotide forms specific double-stranded hybrids

with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gautier et al., Nucl. Acids. Res. 15:6625, 1987). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue et al., Nucl. Acids Res. 15:6131, 1987), or a chimeric RNA-DNA analog (Inoue et al., FEBS Lett. 215:327, 1987).

Oligonucleotides of the invention can be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides can be synthesized by the method of Stein et al. (Nucl. Acids Res. 16:3209, 1988), and methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., Proc. Natl. Acad. Sci. USA 85:7448, 1988).

The nucleic acid molecules should be delivered to cells that express ALARM *in vivo*, e.g., brain, heart, kidney, lung, uterus, endothelial cells, fibroblasts, and bone marrow stromal cells. A number of methods have been developed for delivering DNA or RNA to cells; e.g., molecules can be injected directly into the tissue site, or modified molecules, designed to target the desired cells (e.g., linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be administered systemically.

If intracellular concentrations of the molecule sufficient to suppress translation of endogenous mRNAs are not immediately achieved, a preferred approach uses a recombinant DNA construct in which the oligonucleotide is placed under the control of a strong *pol* III or *pol* II promoter. The use of such a construct to transfect target cells in the patient will result in the transcription of sufficient amounts of single stranded RNAs that will form complementary base pairs with the

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endogenous ALARM transcripts and thereby prevent translation of the ALARM MRNA. For example, a vector can be introduced *in vivo* such that it is taken up by a cell and directs the transcription of an RNA. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired RNA.

Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the RNA can be by any promoter known in the art to act in mammalian, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include, but are not limited to: the SV40 early promoter region (Bernoist et al., Nature 290:304, 1981); the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., Cell 22:787-797, 1988); the herpes thymidine kinase promoter (Wagner et al., Proc. Natl. Acad. Sci. USA 78:1441, 1981); or the regulatory sequences of the metallothionein gene (Brinster et al., Nature 296:39, 1988).

Any type of plasmid, cosmid, YAC, or viral vector can be used to prepare the recombinant DNA construct which can be introduced directly into the tissue site; e.g., the brain, kidney or heart cells. Alternatively, viral vectors can be used that selectively infect the desired tissue (e.g., for brain, herpesvirus vectors may be used), in which case administration can be accomplished by another route (e.g., systemically).

Alternatively, endogenous ALARM gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the ALARM gene (i.e., the ALARM promoter and/or enhancers) to form

triple helical structures that prevent transcription of the ALARM gene in target cells in the body (Helene, Anticancer Drug Des. 6:569, 1981; Helene et al., Ann. N.Y. Accad. Sci. 660:27, 1992; and Maher, Bioassays 14:807, 1992).

Identification of Proteins which Interact with ALARM

The invention also features proteins which interact with ALARM. For example, an ALARM protein or a fusion protein containing ALARM can be used to detect the presence of presenilin 1 in a sample. Any method suitable for detecting protein-protein interactions may be employed for identifying transmembrane proteins, intracellular, or extracellular proteins that interact with ALARM. Among the traditional methods which may be employed are co-immunoprecipitation, crosslinking and co-purification through gradients or chromatographic columns of cell lysates or proteins obtained from cell lysates and the use of ALARM to identify proteins in the lysate that interact with the ALARM. For these assays, the ALARM polypetide can be a full length ALARM, a soluble extracellular form of ALARM or some other suitable ALARM polypeptide. Once isolated, such an interacting protein can be identified and cloned and then used, in conjunction with standard techniques, to identify proteins with which it interacts. For example, at least a portion of the amino acid sequence of a protein which interacts with the ALARM can be ascertained using techniques well known to those of skill in the art, such as via the Edman degradation technique. The amino acid sequence obtained may be used as a guide for the generation of oligonucleotide mixtures that can be used to screen for gene sequences encoding the interacting protein. Screening may be accomplished, for example, by standard hybridization or PCR techniques. Techniques for

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the generation of oligonucleotide mixtures and the screening are well known. (Ausubel, *supra;* and PCR Protocols: A Guide to Methods and Applications, 1990, Innis et al., eds. Academic Press, Inc., New York).

Additionally, methods may be employed which result directly in the identification of genes which encode proteins which interact with ALARM. These methods include, for example, screening expression libraries, in a manner similar to the well known technique of antibody probing of λgt11 libraries, using labeled ALARM polypeptide or an ALARM fusion protein, e.g., an ALARM polypeptide or domain fused to a marker such as an enzyme, fluorescent dye, a luminescent protein, or to an IgFc domain.

The method used to identify the ALARM protein, described below, based on its interaction with presenilin 1 (see also Chien et al., *Proc. Natl. Acad. Sci. USA*, 88:9578, 1991) can also be used to detect other proteins interacting with ALARM. A kit for practicing this method is available from Clontech (Palo Alto, CA).

Briefly, utilizing such a system, plasmids are constructed that encode two hybrid proteins: one plasmid includes a nucleotide sequence encoding the DNA-binding domain of a transcription activator protein fused to a nucleotide sequence encoding an ALARM polypeptide or protein, or an ALARM fusion protein, and the other plasmid includes a nucleotide sequence encoding the transcription activator protein's activation domain fused to a cDNA encoding an unknown protein which has been recombined into this plasmid as part of a cDNA library. The DNA-binding domain fusion plasmid and the cDNA library are transformed into a strain of the yeast *Saccharomyces cerevisiae* that contains a reporter gene (e.g., HBS or *lacZ*) whose regulatory region contains the transcription activator's binding site. Either hybrid

protein alone cannot activate transcription of the reporter gene: the DNA-binding domain hybrid cannot because it does not provide activation function and the activation domain hybrid cannot because it cannot localize to the activator's binding sites. Interaction of the two hybrid proteins reconstitutes the functional activator protein and results in expression of the reporter gene, which is detected by an assay for the reporter gene product.

The two-hybrid system or related methodology may be used to screen activation domain libraries for proteins that interact with the "bait" gene product. By way of example, and not by way of limitation, ALARM may be used as the bait gene product. Total genomic or cDNA sequences are fused to the DNA encoding an activation domain. This library and a plasmid encoding a hybrid of bait ALARM gene product fused to the DNA-binding domain are cotransformed into a yeast reporter strain, and the resulting transformants are screened for those that express the reporter gene. For example, a bait ALARM gene sequence, such as ALARM or a domain of ALARM can be cloned into a vector such that it is translationally fused to the DNA encoding the DNA-binding domain of the GAL4 protein. These colonies are purified and the library plasmids responsible for reporter gene expression are isolated. DNA sequencing is then used to identify the proteins encoded by the library plasmids.

A cDNA library of the cell line from which proteins that interact with bait ALARM gene product are to be detected can be made using methods routinely practiced in the art. According to the particular system described herein, for example, the cDNA fragments can be inserted into a vector such that they are translationally fused to the transcriptional activation domain of GAL4. This library can be co-transformed along with the bait

ALARM gene-GAL4 fusion plasmid into a yeast strain which contains a lacZ gene driven by a promoter which contains GAL4 activation sequence. A cDNA encoded protein, fused to GAL4 transcriptional activation domain, that interacts with bait ALARM gene product will reconstitute an active GAL4 protein and thereby drive expression of the HIS3 gene. Colonies which express HIS3 can then be purified from these strains, and used to produce and isolate the bait ALARM gene-interacting protein using techniques routinely practiced in the art.

In addition, a genetic test can also be used wherein ALARM nucleic acid sequences are used to identify polymorphisms in the ALARM gene which indicate an increased likelihood of developing a condition or disease.

Diagnosis of Diseases Associated with Alterations in ALARM Nucleic Acid Sequences

The invention disclosed herein also relates to diagnosis of various diseases by first identifying the genetic defect in ALARM which causes the disease in question, and then devising an assay using either a hybridization probe or a PCR amplification primer containing the mutant sequence.

After identifying a specific ALARM mutation that is associated with a particular disease, that information can then be used to design an oligonucleotide useful as a diagnostic tool to screen other individuals for that particular disease.

The oligonucleotide can take the form of a hybridization probe or a primer for PCR amplification. Such hybridization probes could range in size from six to 10,000 nucleotides (preferably 13 to 20 nucleotides), while PCR primers could range from ten to 1000 nucleotides (preferably 18 to 25 nucleotides).

If either such screen reveals that the mutation appears in some patients with an autosomal dominant disease but in no unaffected individuals of a statistically significant sample, it can be presumed that the existence of that mutation in the DNA of any tested individual will be informative for the inherited propensity to develop one form of autosomal dominant ALARM-protein related disease. An oligonucleotide which includes the mutant sequence will be useful as a diagnostic tool for screening individuals for that form of the disease. A genetic screening test based on this oligonucleotide, and further including a second oligonucleotide with the normal sequence could be useful not only to detect those homozygous for the mutation (and thus destined to develop the disease), but also those heterozygous for the mutation (and thus carriers of the disease trait).

A genetic screening test can also be used to identify individuals with autosomal recessive ALARM-associated disease, and/or to identify compound heterozygotes. In the latter case, two different mutations, each affecting different copies of the disease gene, are present in the affected patients of a sibship. Each of the two mutations comes from one parent.

Uses of the Invention

The ALARM proteins and nucleic acids of the invention have a variety of uses. For example, an ALARM polypeptide can be used to determine the amount of ALARM-binding presenilin 1 in a sample.

In addition, ALARM antibodies can be used in an immunoassay to monitor the level of ALARM produced by a mammal and also to determine the subcellular location of ALARM in a mammal.

Further, both ALARM polypeptides and ALARM antibodies can be used to identify additional proteins which bind to ALARM.

ALARM nucleic acids can also be used to identify human chromosome 5, as diagnostic agents to identify individuals with mutations in ALARM nucleic acid sequences. In addition, ALARM nucleic acid and polypeptide sequences be used as molecular weight markers and also to block expression of ALARM sequences.

Examples

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims. The following examples illustrate the characterization of human ALARM nucleic acids and polypeptides.

Example 1. Primary Structure of ALARM Nucleic Acids and Polypeptides

The yeast two-hybrid system was used to identify cDNAs from a human brain cDNA library that bind to the Loop region of presenilin 1.

PCR was used to amplify the presenilin 1 loop region, which is defined by EcoRI and BamHI sites at the 5' or 3' ends, respectively and which encodes amino acids 260-400 of presenilin. The PCR products and vector PASE 1 DNA were digested with these two restriction enzymes and then ligated. The resulting construct was confirmed by sequence analysis and named the Loop construct.

Standard procedures were used to identify brain cDNAS that encoded proteins binding to the Loop region. Briefly, the plasmid DNA for the controls of the yeast two hybrid experiment was from the MATCHMAKER II kit (Clontech) which includes pCL1 (full length Gal 4), pVA3 1 (P53 to Gal 4 binding domain), pTD1-1 (SV40 large T-

antigen to activation domain), pLAM 5'-1 (Human lamin C to BD).

Plasmid DNAs were introduced into yeast strain 190, Strain 190 was transformed first with the Loop plasmid and with a human brain cDNA library (the Matchmaker library). In each case, selection for transformants was made on appropriate selective medium.

Plasmid DNA from presumptive colonies containing an interacting cDNA was isolated from a single, well-isolated colony using standard procedures. The plasmid DNA was then transformed into *E. coli*, from which plasmid DNA was prepared.

Using the Loop region as bait, eight colonies were identified in the two hybrid assay. Two colonies were positive upon rescreening, and they were found to carry the identical insert.

To verify that the interaction between the "captured protein" and the Loop region was not an artifact of the two hybrid system, the insert was transcribed and translated *in vitro*, and the resulting protein was tested for its ability to bind a Loop-glutathione S-transferase protein.

Fresh overnight cultures of *E. coli* transformed with pGex-4T-1 or one of its recombinants were diluted 1:10 in LB-Amp and incubated for 2 hours at 37C with shaking until the A_{600} reached 0.6-1.0. IPTG was added to a final concentration of 0.1 mM and the culture was incubated for an additional 3 hours. The cells were washed once with PBS, and resuspended in 1 ml PBS plus protease inhibitors (PMSF, aprotinin, leupeptin, pepstatin) in microfuge tubes and then lysed by mild sonication. Triton X-100 in PBS was then added to a final concentration of 1%. The lysate was rotated at 4°C

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for 20 minutes followed by centrifugation at 14,000 for 10 minutes at 4°C.

The supernatant was rocked for 15-30 minutes at 4°C with 20 μl of 50% (v/v) glutathione-sepharose, which had been previously washed with PBS. After centrifugation, the beads were washed three times with PBS.

In vitro translation was performed using Promega (Madison, WI) TNT kits. Briefly, 1-2 ug of plasmid DNA was mixed with 25 ul of TnT rabbit reticulocyte lysate, 2 ul reaction buffer, 1 ul T7 RNA polymerase, 2 ul amino acid mixture minus methionine, 4 ul ^{35}S-methionine, 1 ul Rnasin and H_2O in a 50 ul reaction volume. The reaction was incubated at 30°C for 2 hours.

In vitro translated proteins were mixed in binding buffer (10 mM Tris-HCl, pH 8.0; 200 mM NaCl; 5 mM EDTA, 0.5% NP-40, 1 mM DTT, 3 mg/ml of BSA, and proteinase inhibitors) with 20 ul of protein A agarose and rocked at 4°C for 1 hour. Antibody was added to the precleared supernatant (1:200), and rocked for 2 hours at 4°C and then 20 ul of protein-A agarose was added and rocked for another 2 hours. The beads were washed then washed 4 times.

Glutathione-sepharose beads bound with GST-fusion proteins were washed with binding buffer (10 mM Tris-HCl, pH 8.0; 200 mM NaCl; 5 mM EDTA, 0.5% NP-40, 1 mM DTT, 3 mg/ml of BSA, and proteinase inhibitors), rocked with aliquots of *in vitro* translated ^{35}S-labeled proteins for 1 hour at 4°C in binding buffer. The beads were washed five times with binding buffer and boiled in sample buffer. The eluted proteins were then analyzed on SDS-PAGE. Binding of the captured protein to the Loop-glutathione S-transferase protein was observed, confirming that the captured protein was not an artifact of the yeast two-hybrid system.

The DNA encoding the captured protein was then sequenced. Sequence analysis was performed using the GCG sequence analysis program.

The captured protein was found to have the DNA sequence shown in Fig. 1, and to encode a protein with the amino acid sequence shown in Fig. 1. The protein contains four copies of a the *arm* repeat, which was originally identified in the *Drosophila melanogaster arm* gene and has been subsequently identified in members of the catenin family. Because members of the catenin family have been associated with the adherens junctions, the new protein has been named ALARM, for adherens-junction linked *arm* protein, or, alternatively, δ catenin. The presence of the *arm* repeats in the ALARM protein, and their similarity to the original *arm* repeat is shown in Fig. 2. The *arm* repeats from the ALARM sequence are represented labeled as i, ii, iii, and iv. Repeat ii is most homologous to *arm*, with 70% homology, while repeat iii is the least homologous, with 31% homology.

Among proteins identified which contain *arm* repeats, ALARM shows the highest homology to pp120, a protein originally identified as a substrate for the tyrosine kinase pp60src (Staddon et al., J. Cell Biol. 130:369, 1995). The pp120 homology is shown in Fig. 3. Overall, ALARM is 60.8 % similar and 43.3% identical to pp120.

The sequence alignment between ALARM and γ catenin is shown in Fig. 4. Overall, ALARM and γ catenin are 52.3% similar and 32.1% identical.

In chromosomal mapping studies, DNA sequences homologous to ALARM-encoding DNA sequences were found to map to chromosome 5.

Example 2. Tissue Localization of ALARM RNA Sequences

To determine the tissues in which ALARM sequences are transcribed, poly A+ RNA was isolated from several human tissues.

RNA was isolated from human tissues using standard procedures. RNA hybridization was performed using Clontech ExpressHyb solution. Briefly, the ExpressHyb Solution was warmed up to 60°C. The nylon membrane was prehybridized in 5 ml of ExpressHyb Solution with continuous shaking at 60°C for 30 minutes. Denatured ALARM DNA (labeled with ^{32}P by random primer extension) was added to 5 ml of fresh ExpressHyb to a final activity 10^6 cpm/ml, and the hybridization was carried out for 1 hour at 60°C. The blot was rinsed in wash solution 1 several times at room temperature for 30-40 minutes with continuous agitation, and then washed in wash solution 2 with continuous shaking at 500C for 40 minutes with one change of fresh solution. The blot was then exposed to x-ray film at -70°C with two intensifying screens.

An intensely hybridizing band 6 kb in size was detected in tissue from brain, as were minor bands of 7 kb and 4.kb. A weak to moderately hybridizing band of 6. kb was detected in the pancreas. Heart tissue gave rise to barely detectable transcripts, and no hybridization was detected in skeletal muscle, kidney, liver, placenta, or lung. These data indicate that ALARM expression is found nearly exclusively in brain tissue.

Example 3. Generation of Antibodies Against ALARM Peptides and Co-Immunoprecipitation Experiments Using ALARM Anti-Sera

Polyclonal anti-ALARM antibodies were raised using standard procedures by injecting a synthetic 14 amino acid peptide having the sequence YETSHYPASPDSWV, corresponding to the 14 carboxy terminal residues of the ALARM protein, into rabbits. Anti-alarm antibodies were also raised against a GST-fusion protein containing the 100 amino terminal amino acids of ALARM as shown in Fig. 1. Antibodies raised to the peptides detected a protein migrating with a size of about 130 kDa.

To determine if ALARM binds cadherin or the β-amyloid precursor protein (βAPP) protein, co-immunoprecipitation experiments were performed in which anti-ALARM sera was used in co-immunoprecipitation experiments using ALARM and each of the respective proteins. Anti-ALARM sera precipitated cadherin protein when ALARM and cadherin proteins were coexpressed *in vitro*. Anti-Alarm anti-sera also immunoprecipitated cadherin in isolated brain tissues. This suggests that ALARM and cadherin interact directly. In addition, because cadherin is found at the adherens junction, it indicates ALARM also localizes to this structure.

Antisera-ALARM sera also co-precipitated the βAPP precursor peptide when these proteins were coexpressed *in vitro*. This result suggests that ALARM and the APPβ protein bind directly, and that ALARM may be involved in generating the Aβ peptide.

Example 4. Cellular Localization of ALARM Polypeptides

Immunolocalization studies examining ALARM expression in neurons cultured from embryonic day 18 rat brains were performed using an anti-ALARM antibody

isolated as described in Example 3. Rat brains showed neuronal staining primarily in the cell body. The observed pattern is consistent with the reported expression pattern of presenilin 1.

Example 5. Diagnostic Assays Utilizing ALARM Hybridization Probes

As described above, a nucleic acid probe containing some or all of the ALARM-encoding sequences of the invention is used to detect ALARM mRNA in a sample of cells (e.g., brain cells) suspected of having altered ALARM expression. The probe used is a single-stranded DNA or RNA (preferably DNA) antisense to the ALARM coding sequence. It is produced by synthetic or recombinant DNA methods, and labelled with a radioactive tracer or other standard detecting means. The probe includes from 15 up to the full ALARM coding sequence, and preferably is at least 30 nucleotides long. The assay is carried out by standard methods of *in situ* hybridization or Northern analysis, using stringent hybridization conditions. Control hybridization assays are run in parallel using normal cells or tissue sections from the same type of tissue as the test sample, and/or cells from a known tissue or cell line, or a tissue section, whose ALARM transcription levels are known. Cells which exhibit an altered level of hybridization to the probe, compared to the level seen with normal epithelial cells, are likely to be indicative of a neurological condition. The amount of hybridization is quantitated by standard methods, such as counting the grains of radioactivity-exposed emulsion on an *in situ* hybridization assay of a biopsy slide, or by densitometric scan of a Northern blot X-ray film. Alternatively, comparison of the test assay results with the results of the control assays is relative rather than

quantitative, particularly where the difference in levels of hybridization is dramatic.

Example 6. Diagnostic Assays Utilizing Alarm Antibodies

Antibodies specific for ALARM are generated by standard polyclonal or monoclonal methods, using as immunogen a purified, naturally-occurring ALARM; recombinant ALARM; or any antigenic fragment of ALARM (e.g., the peptides described above) which induces antibodies that react with naturally-occurring ALARM. The latter fragment can be produced by synthetic or recombinant methods, or by proteolytic digestion of ALARM. If desired, the antigenic fragment is linked by standard methods to a molecule which increases the immunogenicity of the fragment, such as keyhole limpet hemocyanin (as described above). The polyclonal or monoclonal antibodies so produced are screened using purified recombinant or naturally occurring ALARM, or as described above, to select those which form an immunocomplex with ALARM specifically.

The antibodies so produced are employed in diagnostic methods for detecting cells, tissues, or biological fluids in which the presence of ALARM is altered relative to normal cells, an indication that the patient has a neurological condition. The sample tested may be a fixed section of a tissue biopsy, a preparation of cells obtained from a suspect tissue, or a sample of biological fluid, such as cerebrospinal fluid. Standard methods of immunoassay may be used, including those described above as well as sandwich ELISA. If the tested cells express altered levels of ALARM protein in this assay relative to normal cells of the same tissue type, the tested cells are likely to represent a neurological condition. The anti-ALARM antibodies are also used to

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detect alterations in the levels or ALARM-binding activity of other cellular components, e.g., presenilin-1, cadherein, or βAPP protein, that interact with ALARM. Anti-ALARM anti-bodies are used to detect these proteins using co-immunoprecipitation assays known in the art.

Example 7. Screens For and Uses of Therapeutic Agents Based on their Interaction with ALARM

Cells in which the expression or activity of the endogenous ALARM gene is altered, i.e., down-regulated, are used as a screening tool to identify compounds or treatment strategies that increase expression or activity of the ALARM gene.

The cells are treated *in vitro* with the candidate compounds, and the amount of ALARM expression is determined using either a hybridization assay (e.g., Northern analysis) or an immunoassay. If a given compound is found to increase ALARM expression, it is then further tested to see whether treatment with the compound prevents the development of a neurological condition *in vivo* in an appropriate animal model. An appropriate animal model is a transgenic model constructed using the techniques described above in which a ALARM gene is expressed under the control of an inducible promoter.

A compound effective both in increasing ALARM expression or activity (e.g., in facilitating its binding to presenilin 1 or βAPP) is a potential therapeutic useful for the treatment of conditions in which ALARM expression is increased compared to normal cells. Further evaluation of the clinical usefulness of such a compound follows standard methods of evaluating toxicity and clinical effectiveness of agents for treating neurological conditions.

Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is:

1. A substantially pure ALARM polypeptide.

2. The polypeptide of claim 1, wherein said polypeptide comprises an amino acid sequence that is at least 85% identical to the amino acid sequence shown in Fig. 1.

3. The polypeptide of claim 1, wherein said polypeptide comprises an amino acid sequence that is at least 95% identical to the amino acid sequence shown in Fig. 1.

4. The polypeptide of claim 1, wherein said polypeptide comprises the amino acid sequence shown in Fig. 1.

5. The ALARM polypeptide of claim 1, wherein said polypeptide comprises an antigenic determinant of human ALARM.

6. An isolated nucleic acid encoding an ALARM polypeptide.

7. The isolated nucleic acid molecule of claim 6, wherein said molecule comprises a nucleotide sequence encoding a polypeptide having a sequence that is at least 85% identical to the sequence of the human ALARM polypeptide shown in Fig. 1.

8. The nucleic acid molecule of claim 7, wherein said nucleotide sequence encodes a polypeptide that binds presenilin-1.

9. The nucleic acid molecule of claim 6, wherein said molecule encodes the polypeptide shown in Fig. 1.

10. The isolated nucleic acid molecule of claim 6, said molecule comprising the nucleotide sequence of shown in Fig. 1.

11. The isolated nucleic acid molecule of claim 6, said molecule hybridizing under stringent conditions to the nucleic acid sequence shown in Fig. 1.

12. A vector comprising the recombinant nucleic acid of claim 6.

13. A cell comprising the recombinant nucleic acid of claim 6.

14. An antibody which selectively binds to an ALARM polypeptide of claim 1.

15. An antibody produced by administering an ALARM polypeptide according to claim 1 to a mammal.

16. An antibody produced by administering an immunogenic fragment of an ALARM peptide to a mammal.

17. A method of inhibiting expression of an ALARM gene comprising administering to a cell containing an ALARM transcript an anti-sense ALARM oligonucleotide.

18. A method of detecting presenilin 1 in a sample comprising contacting said sample with the polypeptide of claim 1.

19. The method of claim 18 wherein said sample is from a human.

20. The method of claim 19 wherein said sample is from cerebrospinal fluid.

21. A method of diagnosing in a human subject a disease in which a mutant form of a protein which interacts with ALARM is a causative agent, said method comprising analyzing a sample of fluid from said human subject to determine the presence or absence of said ALARM-interacting protein.

22. A method of detecting an ALARM-containing complex in a biological sample, comprising contacting said sample with the ALARM protein of claim 1 and determining whether said ALARM protein binds to a component of said sample.

23. A method of detecting an ALARM-containing complex in a biological sample, comprising contacting said sample with or the antibody of claim 14 and determining whether said ALARM antibody binds to a component of said sample.

24. A method of diagnosing an altered level of presenilin 1 in a sample by contacting the sample with ALARM and determining whether the sample contains presenilin 1 that binds to ALARM.

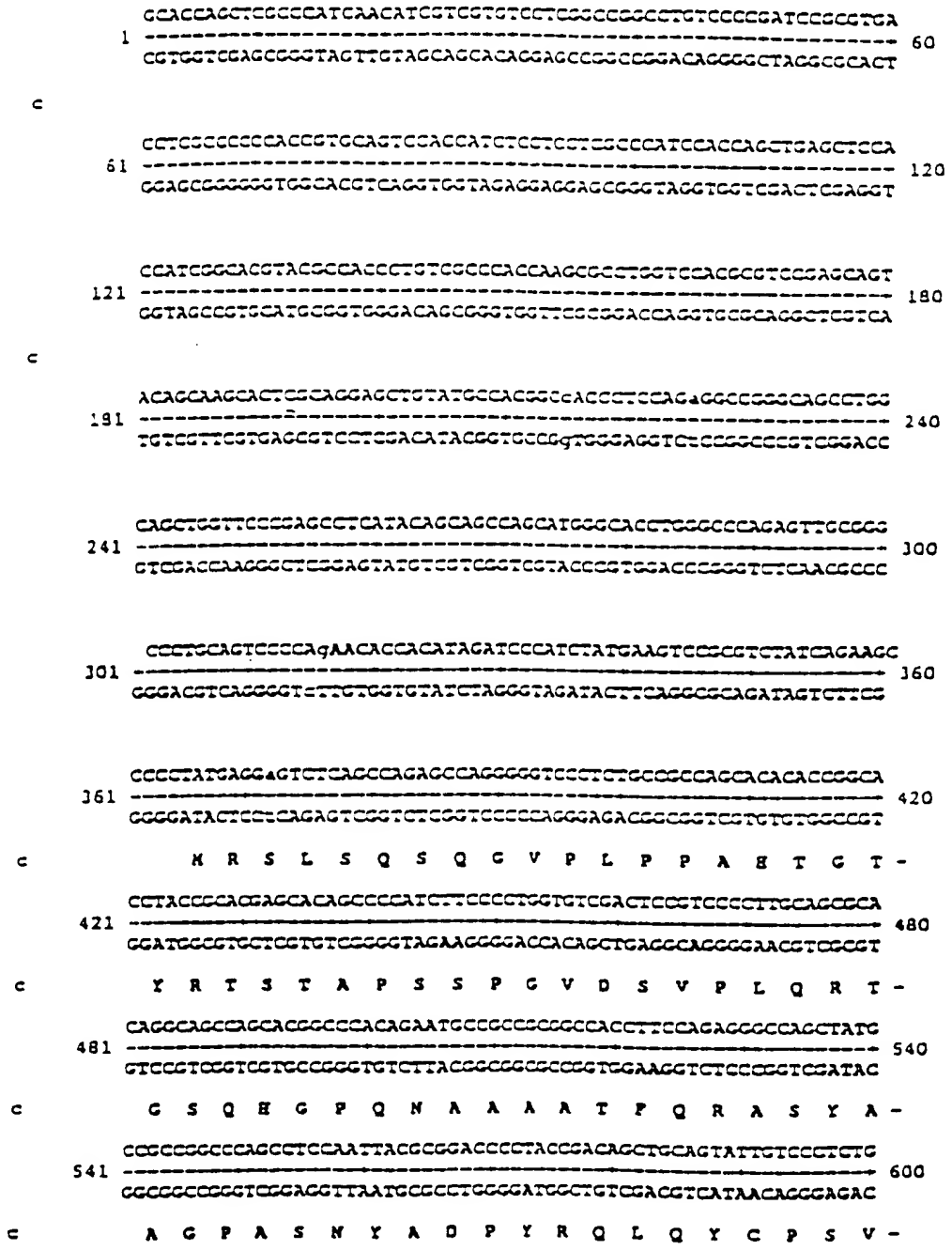

Fig. 1A

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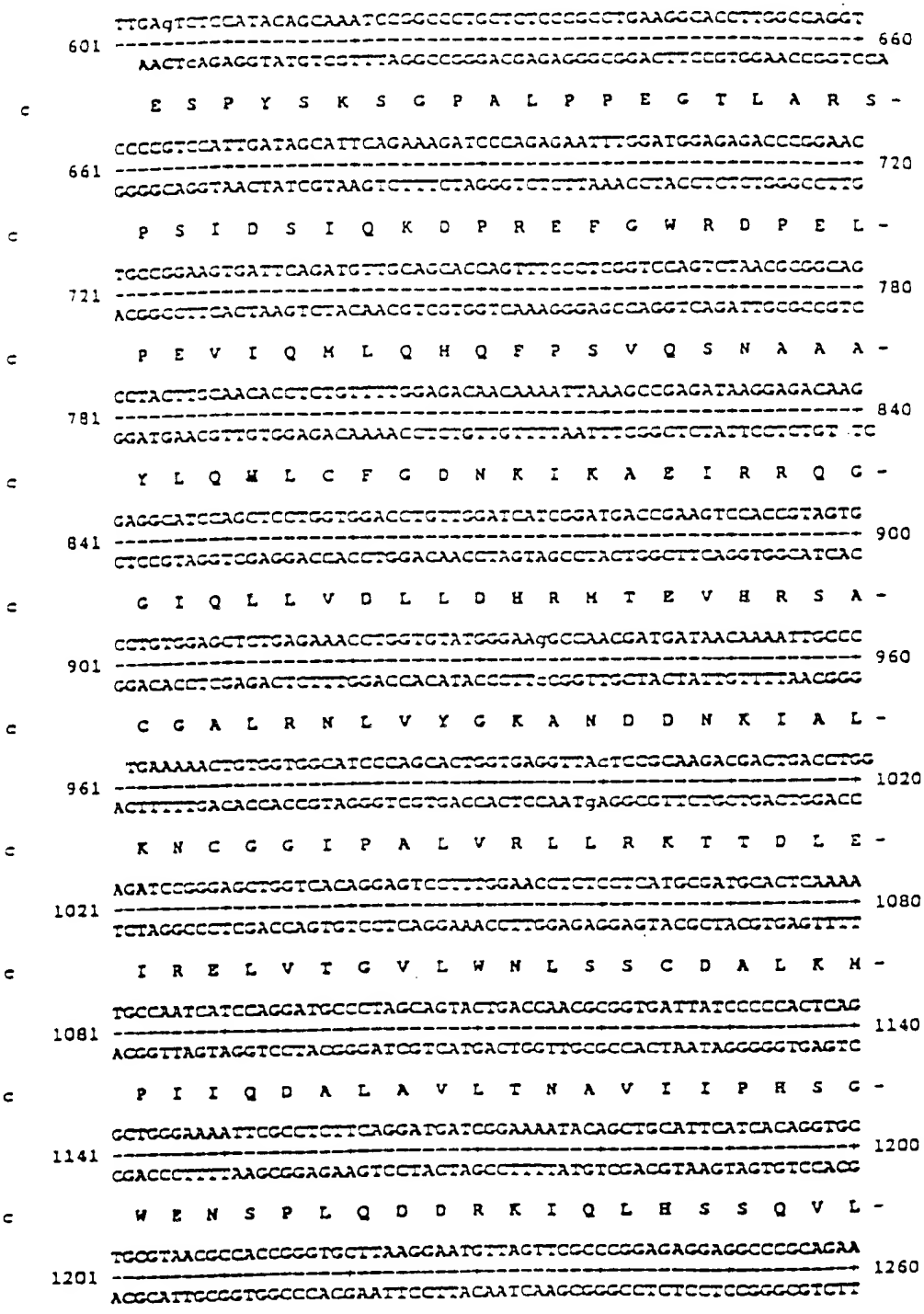
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721
780
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840
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1141
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Fig. 1B

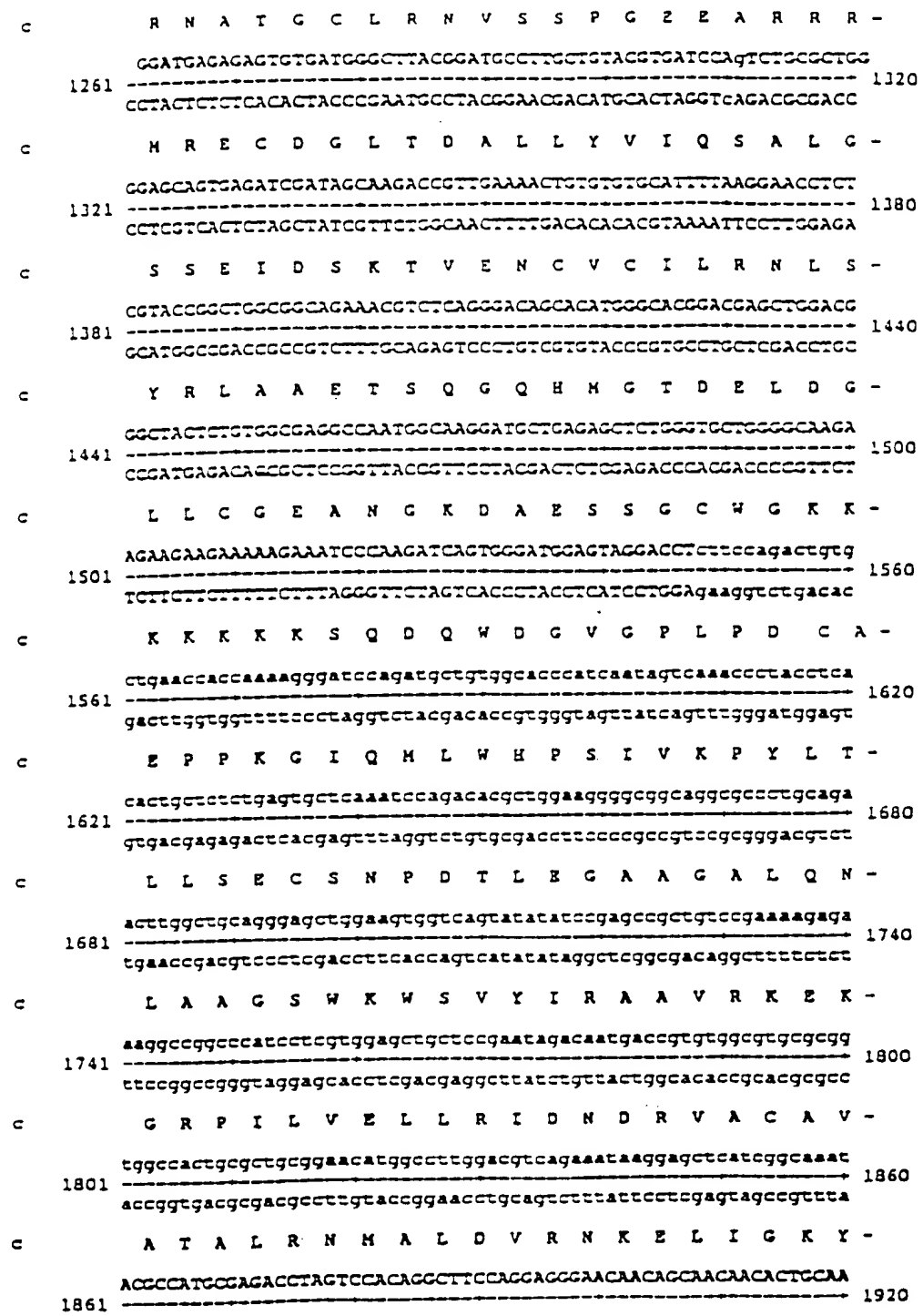

Fig. 1C

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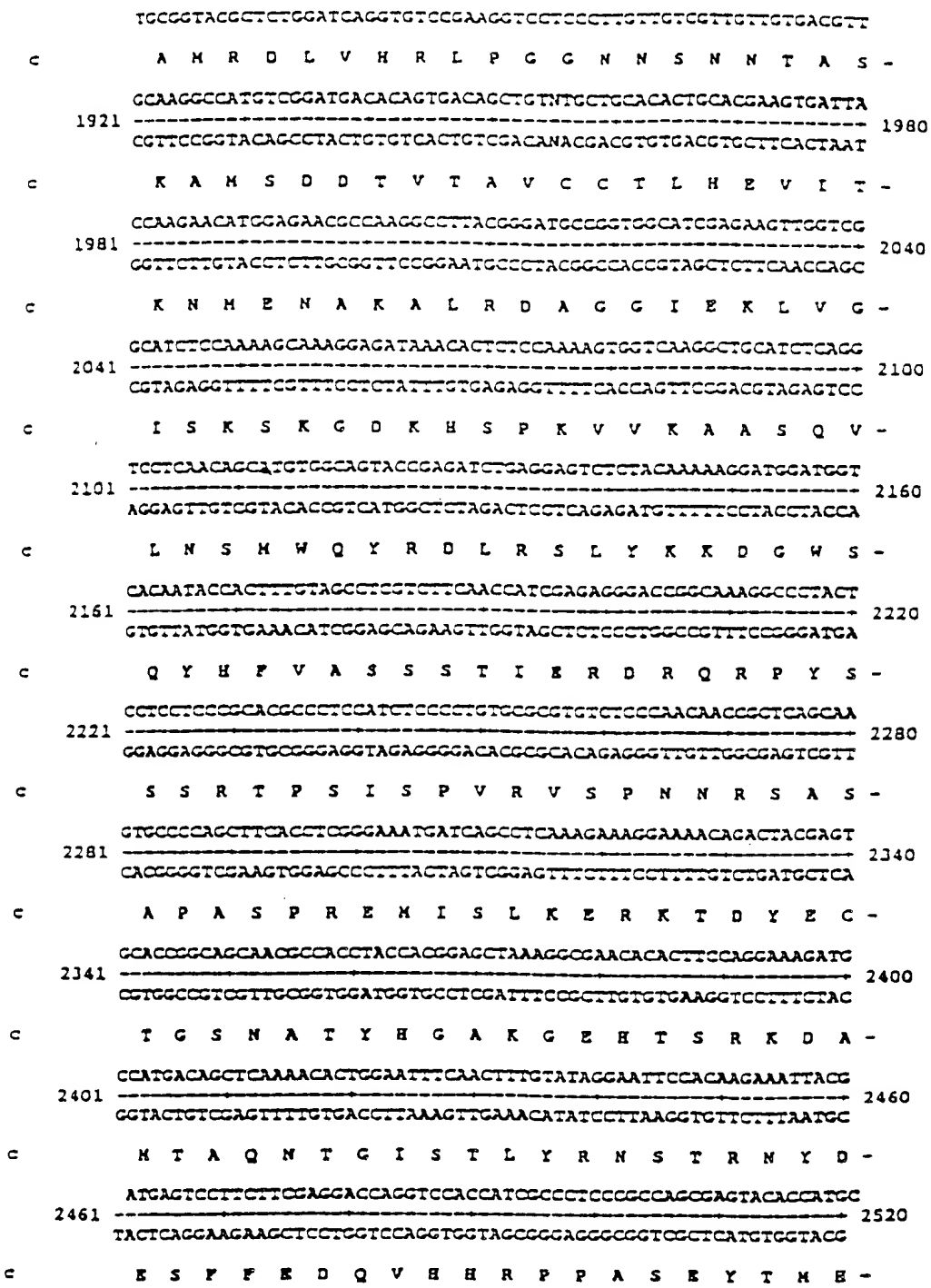
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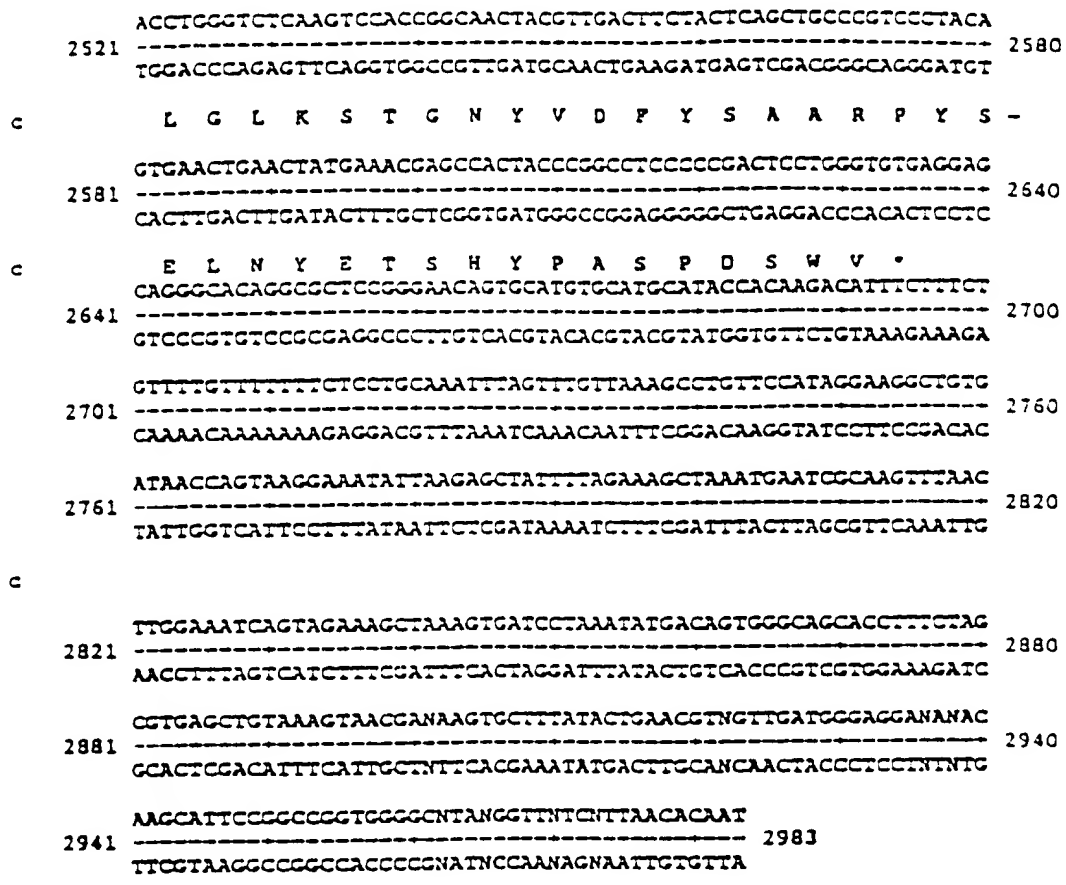
Fig 10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2881 2940
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2941 2983
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Fig 1F

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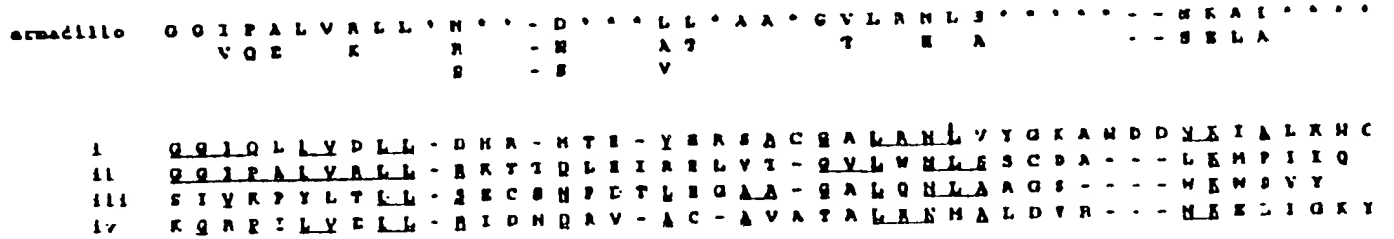
Homology to repeated regions in armadillo, γ-catenin and β-catenin



Homologous to consensus sequences:

i: 67%
ii: 70%
iii: 31%
iv: 59%

*: non-consensus sequence
-: gap

This alignment is essentially similar to that in a paper published in Oncogene (1993), 7: 2439-2445

Fig. 2

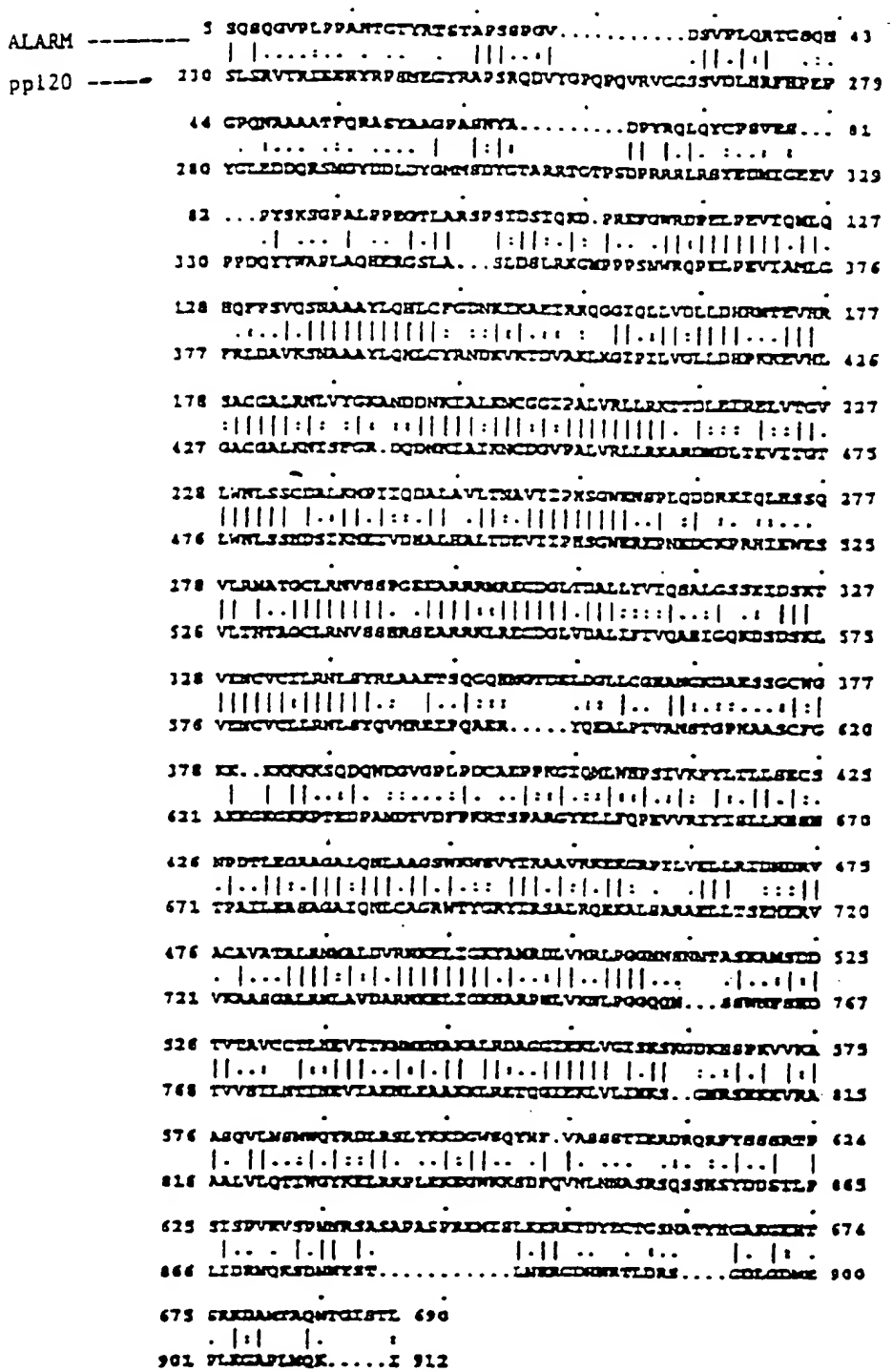


Fig. 3

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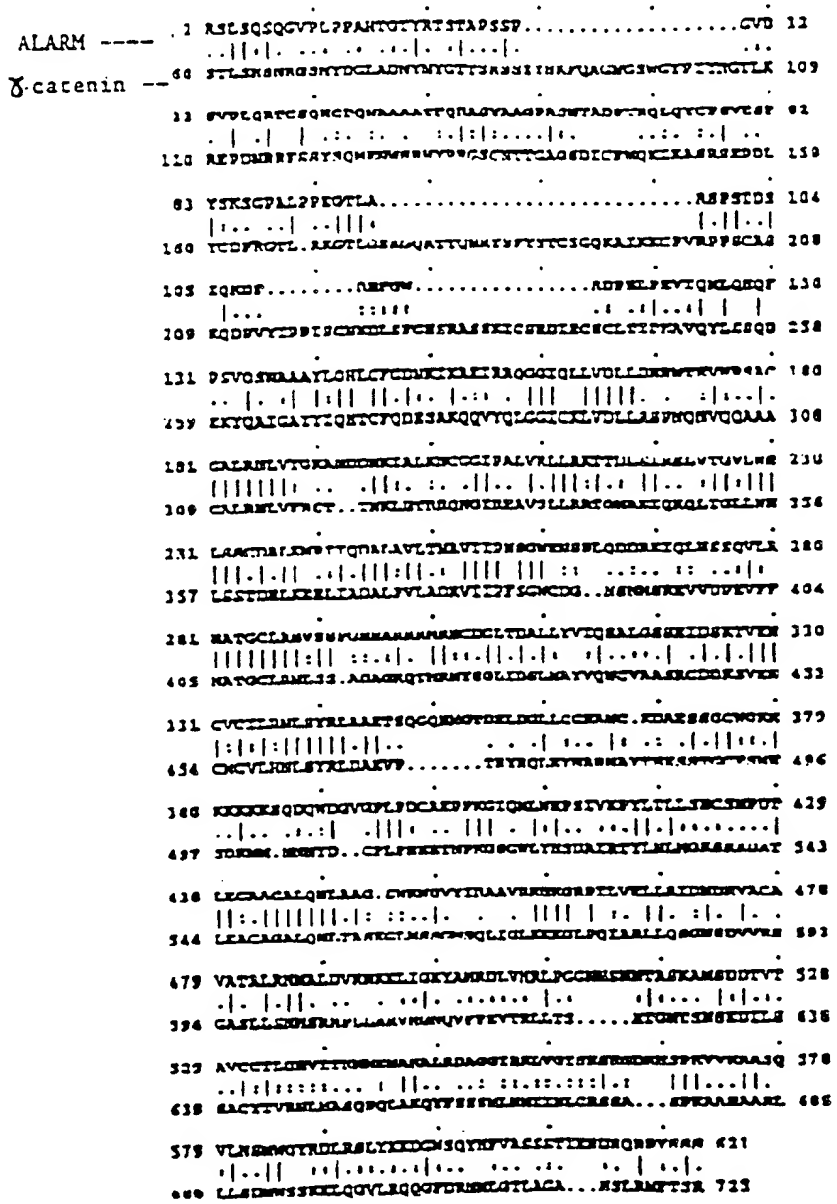

Fig. 4

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/22093

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 33/53; C07K 1/00, 16/00; C07H 21/02, 21/04
US CL : 435/7.1, 7.8; 530/350, 387.1, 387.9; 536/23.1, 23.5, 24.1, 24.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 7.8; 530/350, 387.1, 387.9; 536/23.1, 23.5, 24.1, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS, DIALOG, MEDLINE, BIOSIS, EMBASE
search terms: ALARM, catenin, adherens-junction, arm protein, armadillo protein, presenilin

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	McCREA et al. A homolog of the armadillo protein in Drosophila (plakoglobin) associated with E-cadherin. Science. 29 November 1991, Vol. 254, pages 1359-1361, see entire document.	1-5
Y	RUBINFELD et al. The APC protein and E-cadherin form similar independent complexes with alpha-catenin, beta-catenin, and plakoglobin. Journal of Biological Chemistry. 10 March 1995, Vol. 270, No. 10, pages 5549-5555, see entire document.	1-5, 14-16, 21
Y	TAO et al. Beta-catenin associates with the actin-bundling protein fascin in a noncadherin complex. Journal of Cell Biology. September 1996, Vol. 134, No.5, pages 1271-1281, see entire document.	1-5, 14-16, 22, 23

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search 04 MARCH 1998	Date of mailing of the international search report 20 MAR 1998
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INTERNATIONAL SEARCH REPORT

International application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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